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# Adsorption of Cyanophage AS-1 to Unicellular Cyanobacteria and Isolation of Receptor Material from *Anacystis nidulans*

BEHDAD SAMIMI AND GERHART DREWS*
*Institute of Biology 2, Albert Ludwigs University, D-7800 Freiburg i. Br., West Germany*

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Cells of unicellular cyanobacteria of typological group Ia, containing approximately 50 mol% guanine + cytosine (G+C) in their DNA (R. Y. Stanier, R. Kunisawa, M. Mandel, and G. Cohen-Bazire, Bacteriol. Rev. 35:171-205, 1971), were susceptible to infection by the cyanophage AS-1. Cyanobacteria of the same typological group, containing approximately 65 mol% G+C in their DNA, did not adsorb the cyanophage AS-1 or adsorbed it at a low rate. AS-1 was not propagated by any of the investigated strains with a high G+C content in their DNA. However, cells of strains 6907 and 6911 were lysed by cyanophage AS-1. A comparison of the host range of this phage with the lipopolysaccharide composition of host and non-host cell walls suggests that lipopolysaccharides are involved in the adsorption process. About 8 μg of lipopolysaccharide per ml from host strains inactivated 50% of the particles of a solution containing 100 PFU/ml after 60 min of incubation at 30°C. Material with receptor activity was extracted from the host strain *Anacystis nidulans* KM. The extract was purified of glycolipids and pigments, and a fraction showing receptor activity was isolated. This fraction contained three polypeptides of molecular weights between 54,000 and 64,000. Heat and protease treatment of whole cells and of isolated receptor material decreased the receptor activity. The fluorescence intensity of *A. nidulans* cells labeled with 1-anilino-8-naphthalene sulfonate was increased when AS-1 was adsorbed to these cells. The participation of lipopolysaccharides and proteins in the formation of the receptor complex is discussed.

AS-1 is a large cyanophage that infects cells of *Synechococcus* strains 6301, 6908, and 6311 (28). The morphology, infection, and intracellular replication of AS-1 and AS-1M have been described in recent reports (26, 28, 32-34). In the course of infection, the virus attaches to the host cell with its tail (26). The reaction between phage tail and specific attachment sites (receptors) on the cell surface is thought to involve a series of steps finally leading to irreversible adsorption (9, 24). Numerous bacteriophages such as ε15, P22, φX174, T3, T4, and T7 are specifically adsorbed by lipopolysaccharides (LPS) exposed on the cell surface of their hosts (24, 31). The receptors for T2 and T6 phages are proteins (39). It has been proposed that the T5 receptor consists of protein and LPS (39). There is some evidence indicating that the active part of the T5 receptor is a protein with a molecular weight of 85,000 (10). Attachment sites on the cell surface seem to be bound to the macromolecular structures that join outer membrane and cytoplasmic membrane at specific sites (5-7).

Cell walls of cyanobacteria have the same fine structure as is found in gram-negative bacteria (3, 11, 15, 16), although the basic electron-dense layer of cyanobacterial cell walls is somewhat thicker than the corresponding layer of gram-negative bacterial cell walls (3, 15, 16, 40). Peptidoglycan (murein), protein, and LPS have been identified in the cell walls of cyanobacteria (11, 13, 18, 20, 37, 40). The LPS composition of most of the *Anacystis-Synechococcus* strains of typological group Ia (32), which were used in the present studies, has been identified (12, 20, 40; unpublished data). Schnayer and Jenifer (Proc. Am. Phytopathol. Soc. 1:144, 1974) have shown for the first time that the cyanovirus AS-1 can be inactivated by LPS isolated from cyanobacteria. This paper describes the composition of the cyanophage AS-1 receptor present on the cell walls of the cyanobacterium *Anacystis nidulans*. It will be shown that LPS as well as proteinaceous material of the cell wall inactivate the cyanophage AS-1.

## MATERIALS AND METHODS

**Strains.** The cyanophage AS-1 strain used throughout these studies was obtained from R. Safferman (Cincinnati, Ohio). The virus stock was prepared by repeated single-plaque isolation. The following strains of cyanobacteria were used: *A. nidulans* KM

(22), obtained from N. Carr, Liverpool; *Anacystis* strains IUCC 625, IUCC 1549, and IUCC 1949 and *Synechococcus cedrorum* IUCC 1191, obtained from R. Haselkorn, Chicago, Ill. (IUCC = Indiana University Culture Collection). The following strains of typological group Ia of unicellular cyanobacteria were obtained from R. Y. Stanier, Paris: 6311, 6301, 6307, 6910, 6908, 6603, 6312, 6907, and 6911. All cultures were started from single colonies.

**Culture techniques.** Cyanobacteria were cultivated in 500-ml Erlenmeyer flasks at 28°C under constant stirring, using the medium described by Allen (2). Large-scale production was carried out at 30°C in aerated (2% [vol/vol] $CO_2$ in air) carboys. The cultures were illuminated with cool white fluorescent lights. For production of virus stocks, the host cells were grown to a concentration of $2 \times 10^8$ cells/ml and infected in the exponential growth phase with virus at a multiplicity of infection (MOI) of 0.1. Lysis was observed beginning at about 8 h after addition of phages (decrease in optical density). Phage particles were harvested approximately 16 h later. The number of viable cells was estimated from the number of colony-forming cells by plating. The total cell count was calculated from turbidimetric measurements and microscopic determination in a Thoma chamber (optical density at 750 nm of 1.0 corresponded to $0.8 \times 10^9$ cells of *Anacystis* IUCC 625/ml).

**Purification of cyanophage AS-1.** Crude lysate was spun in a Padberg continuous-flow centrifuge (Padberg K.G., Lahr, West Germany) to remove cell debris. The titer of the supernatant was approximately $0.5 \times 10^9$ PFU/ml. The crude phage suspension was then concentrated by low-pressure evaporation in dialysis tubing to about $2.5 \times 10^{10}$ PFU/ml and purified by ammonium sulfate precipitation (30%, wt/vol) followed by ultracentrifugation. The precipitate was dialyzed against TMN buffer (see below) and centrifuged (5,000 × *g*, 15 min). The titer in the supernatant after centrifugation was $4.6 \times 10^{11}$ PFU/ml. A further purification was obtained by CsCl buoyant density gradient centrifugation, the phage particles banding at a density of $\rho_{22°C} = 1.50$. The titer of the material in the band was $1.15 \times 10^{12}$ PFU/ml. The buffer solution used for storage and routine experimental work contained 0.01 M Tris buffer, adjusted with HCl to pH 7.5, 1 mM $MgCl_2$, and 10 mM NaCl. This buffer is referred to herein as TMN buffer.

**Determination of phage activity.** The titer of active virus particles was estimated by the agar double-layer plaque technique (1) modified by Safferman and Morris (29).

Adsorption rates of AS-1 to cyanobacteria were determined by methods described elsewhere (1, 21, 28). The cell concentration was adjusted to $2.5 \times 10^9$ cells/ml. Cells were infected with cyanophage AS-1 at a multiplicity of 0.01, and the mixture was incubated in a shaking water bath at 30°C. At various times, 0.1-ml samples were withdrawn, diluted 100 times with TMN buffer, and centrifuged for 10 min at 5,000 × *g* at 4°C. The supernatant was assayed for free viruses (PFU per milliliter).

The inactivation of AS-1 by receptor material was measured after incubation of AS-1 in TMN buffer (final concentration, $10^3$ PFU/ml) with various concentrations of receptor material at 30°C for 120 min. The samples were diluted and centrifuged (5,000 × *g*, 10 min); 0.1 to 0.2 ml of the supernatant was mixed with 1 ml of cells of *A. nidulans* KM ($1.5 \times 10^9$ to $2.0 \times 10^9$ cells/ml) and incubated at 30°C for 1 h. After incubation, samples were mixed with 2.5 ml of soft-agar (0.75% Difco agar) medium and plated on solid medium to estimate the number of PFU per milliliter.

The lytic activity of AS-1 on the different strains of unicellular cyanobacteria was estimated by the drop test. One milliliter of cyanobacteria ($2 \times 10^9$ to $3 \times 10^9$ cells/ml) was mixed with 2.5 ml of soft agar and poured into plates containing bottom agar (1.5% agar). Four drops of a purified AS-1 suspension (0.05 ml each, of the following concentrations: $2 \times 10^5$, $10^6$, $10^8$, and $2 \times 10^9$ PFU/ml) were placed on the solidified agar.

The efficiency of plating of AS-1 with different host strains in relation to *Synechococcus* 6311 was measured in the following way: 3 ml of cyanobacteria ($2 \times 10^9$ cells/ml) was mixed with 0.1 ml of AS-1 and incubated for 1 h at 30°C. One milliliter of this suspension was mixed with 2.5 ml of soft agar and poured into a plate containing bottom agar. The concentration of cyanophage AS-1 was varied from $8 \times 10^4$ to 80 PFU/ml in dilution steps of 10. Each concentration of AS-1 was tested with cyanobacterium strain 6311 in comparison with other strains.

**Isolation and purification of the receptor complex from *A. nidulans* KM.** The receptor complex was separated from cells by mild alkaline treatment (10). The alkaline extract was neutralized, and the receptor material was precipitated with ammonium sulfate. The precipitate of 20% saturation was discarded, and active material precipitated from the supernatant at 75% saturation of $(NH_4)_2SO_4$. The precipitate was dialyzed, concentrated by low-pressure evaporation, and chromatographed on Bio-Gel A50. The column (90 by 3 cm) was equilibrated and eluted with 10 mM sodium phosphate buffer, pH 7.0. The active fractions from Bio-Gel chromatography were pooled, concentrated, dialyzed as described, and applied to a Whatman DEAE (D52)-cellulose column (50 by 2.5 cm). The fractions were eluted with 10 mM Tris buffer, pH 7.5, plus 2% Triton X-100 and increasing concentrations of NaCl (10) from 0.1 to 1.0 M. Active fractions were pooled, precipitated at 4°C with ethanol (precooled to −20°C), dialyzed against 50 mM Tris buffer, and lyophilized. Some samples were subjected to high-voltage electrophoresis (1,000 to 1,500 V) on Cellogel (cellulose acetate) strips (17, 30). Extraction of receptor material with chloroform-methanol (27) enriched the receptor in the methanol phase.

**Other methods.** LPS were extracted with hot phenol-water and purified by ultracentrifugation of the aqueous phase (20, 37). The total content of amino acids in this preparation was less than 0.5% (20). The proteins of the receptor material were separated by polyacrylamide gel electrophoresis in 10% gels (23).

**Treatment of receptor complex with trypsin.** A 500-μg/ml amount of purified receptor complex in 10 mM Tris-hydrochloride buffer (pH 7.7) was mixed with 500 μg of trypsin per ml and incubated at 30°C for 2 h. The reaction was terminated by addition of phenylmethylsulfonyl fluoride (0.5 mg in 0.5 ml of

ethanol). The sample was centrifuged, dialyzed, diluted, and incubated with AS-1 (100 μg of receptor plus 1,000 PFU/ml) for 120 min at 30°C.

**Fluorescence labeling.** Five milliliters of cyanobacteria suspension ($0.8 \times 10^9$ cells/ml) in 0.9% NaCl solution was mixed with 50 μl of $6 \times 10^{-5}$ M 1-anilino-8-naphthalene sulfonate (ANS). After 15 min the cells were centrifuged and resuspended in NaCl solution to $4 \times 10^8$ cells/ml. Fluorescence of samples was measured in a Perkin-Elmer fluorescence spectrophotometer at 35°C. Excitation and emission wavelengths were 360 and 450 nm, respectively.

## RESULTS

**Host range and adsorption experiments.** The adsorption of cyanophage AS-1 to the host *A. nidulans* is a relatively slow process (Table 1), apparently following the kinetics of a first-order reaction (28; Fig. 1). The adsorption rate is temperature dependent. Under standard conditions (TMN buffer, 0.01 M NaCl, 60 min) 5% of AS-1 phages were adsorbed at 4°C, 30% at 15°C, 48% at 22°C, 50% at 30°C, and 57% at 37°C. A temperature increase from 37 to 47°C causes decrease in the adsorption rate due to inactivation of cyanophage (Fig. 2). The adsorption rate increases with an increasing NaCl concentration (Table 2). The burst size is not correlated with the NaCl concentration but varies with the strain.

All host strains examined belong to typological group Ia and have DNA with a guanine plus cytosine (G+C) content of approximately 50 mol% (35). Cells of certain strains having a DNA with a somewhat higher moles percent G+C adsorbed cyanophage AS-1 at a low rate (Fig. 1; Table 2). AS-1 phage does not lyse cells of these strains and is not propagated in these organisms. Strains 6907 and 6911 adsorb the virus at a rate of about 30% (Table 2). When cultures of these strains are infected by AS-1 at an MOI of 1.0, the optical density of the culture decreases relative to the control culture. After infection at an MOI of 10, the culture is lysed within 24 h, but the number of PFU is not increased, indicating that the cells of these strains may adsorb virus particles and subsequently undergo lysis, however, without producing active cyanophage progeny. *Synechococcus* strain 6312 has a DNA with a G+C composition similar to those of host strains but does not adsorb AS-1. This strain, however, differs in other respects from the other strains of the group (35). Strains 6714 and 6803 (high G+C) and 6308 and 6807 (low G+C ratio) of typological group IIA (*Aphanocapsa*) do not adsorb and propagate AS-1 (data not shown). The efficiency

TABLE 1. *Adsorption of cyanophage AS-1 to cyanobacteria*[a]

| Host strain | Adsorption (%) after incubation for: | | | |
|---|---|---|---|---|
| | 15 min | 30 min | 45 min | 60 min |
| *A. nidulans* IUCC 625 | 18 | 44 | 56 | 73 |
| *S. cedrorum* IUCC 1191 | 17 | 24 | 51 | 67 |
| *Anacystis* sp. IUCC 1549 | 11 | 17 | 33 | 51 |

[a] Incubation in culture medium containing 0.05 M NaCl at 30°C.

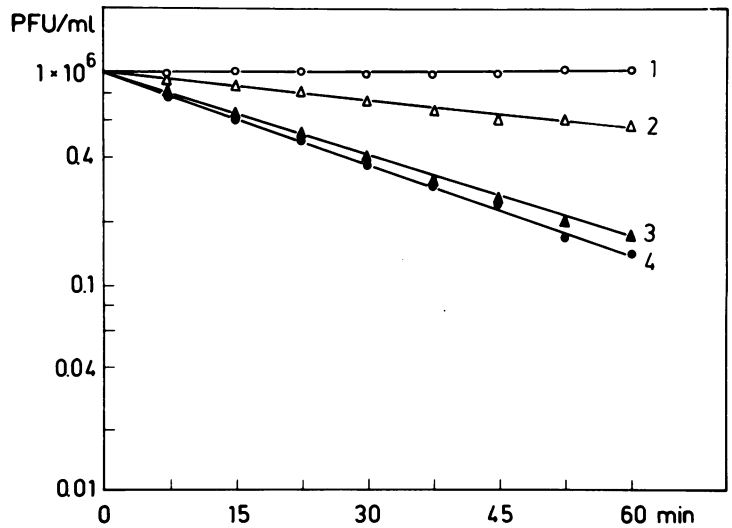



FIG. 1. *Kinetics of adsorption of cyanophage AS-1 to unicellular cyanobacteria. Ordinate: Number of cyanophages per milliliter not adsorbed by cyanobacteria (PFU per milliliter) after various periods of incubation (abscissa) of cyanophage AS-1 (MOI, 0.01) with cyanobacteria. Cyanobacteria strains: 1, 6312; 2, 6907; 3, 6311; and 4, A. nidulans KM.*

of plating within the group of host strains varied between 40 and 100% (Table 2).

The adsorption rate of AS-1 to a specific host strain was found to be approximately constant during the exponential growth phase, but was lower when cells of the stationary phase were infected.

**Possible role of cell wall proteins in the adsorption process.** The participation of proteins of the host cell wall in the process of infection by phage AS-1 is suggested by the following observations. Four major and some minor protein bands were detected in polyacrylamide gel electrophoresis of cell wall preparations from *A. nidulans* (15). The adsorption rate of AS-1 to cells of *A. nidulans* KM was reduced by approximately 50% when these cells were heated in TMN buffer at 60°C for 60 min before the cyanophages were added. A pretreatment of cells with proteases likewise reduced the ability of these cells to adsorb AS-1 (Fig. 3). Pronase pretreatment reduced the adsorption rate by 80%; trypsin pretreatment (followed by the addition of trypsin inhibitor) brought about a reduction of 85%. Traces of active protease that might be remaining on the cell surface after washing and dilution were assumed to be less than 2 μg/ml. This concentration inactivates AS-1 by less than 10% (Table 3).

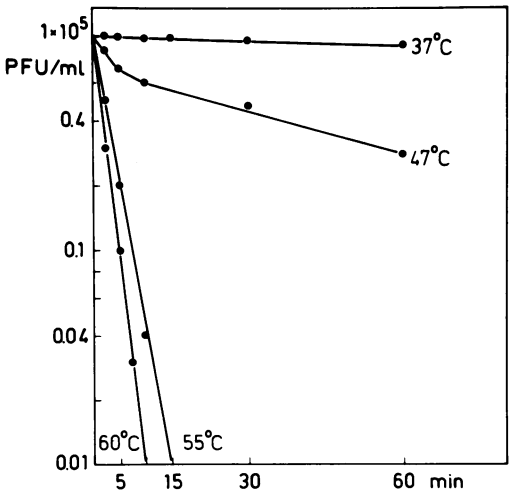



FIG. 2. *Influence of temperature on infectivity of cyanophage AS-1. The number of PFU per milliliter was estimated after incubation of cyanophage in TMN buffer for various times (abscissa) at different temperatures.*

The attachment of phage AS-1 to proteins of the cell wall may induce changes in the conformational state of the cell surface. The fluorescent dye ANS has been used as an indicator for conformational changes in membranes (4, 14, 19, 36, 38). An increase of fluorescence intensity was observed when cells of *Escherichia coli* B, labeled with the probe ANS, were infected by bacteriophage T4 (8). Similarly, the fluorescence intensity of cells of *A. nidulans* KM, labeled with ANS, increased immediately after addition of AS-1 (Fig. 4). The addition of 2.5 cyanophages per cell increased the intensity to 50% of the enhancement after addition of 20 PFU/cell (Fig. 4A). Twenty PFU/cell effected maximum en-

TABLE 2. *Adsorption and lytic activity of cyanophage AS-1*[a]

| Strain | DNA G + C (mol %)[b] | Adsorption (%) after 60 min in presence of: | | Burst size (0.1 M NaCl) | EOP (%) | Lytic activity |
|---|---|---|---|---|---|---|
| | | 0.01 M NaCl | 0.1 M NaCl | | | |
| *A. nidulans* | | | | | | |
| KM | | 58 | ND | ND | 98 | ++ |
| 6301 | 55.1 | 55 | 92 | ND | 86 | ++ |
| 6311 | 55.1 | 59 | 95.6 | 63 | 100 | ++ |
| 6908 | 55.6 | 51 | 85.6 | 51 | 60 | ++ |
| *Anacystis* IUCC 1549 | | 38 | 67.3 | 32 | 42 | ++ |
| *Synechococcus* | | | | | | |
| 6910 | 48.0 | 51.8 | 84.5 | 51 | 42 | ++ |
| 6312 | 50.2 | 1.2 | 2.8 | 0 | 0 | − |
| *C. peniocystis* | | | | | | |
| 6307 | 69.7 | 0.7 | 2.7 | 0 | 0 | − |
| 6603 | 65.7 | 2.0 | 6.6 | ND | 0 | − |
| *Synechococcus elongatus* 6907 | 71.4 | 25 | 34 | 0 | 0 | + |
| *Microcystis aeruginosa* 6911 | 66.3 | 17.5 | 26.4 | 0 | 0 | + |

[a] EOP, Efficiency of plating (percentage of plaques that appear on a particular host as a fraction of the number that appear on the most efficient host) (60-min incubation; MOI, 0.01). Lytic activity: ++, cells are lysed and AS-1 propagated; +, cells are lysed, but cyanophages are not propagated; −, no lysis. The results are means of three experiments. The error does not exceed 5%. ND, Not determined.

[b] From reference 35.

hancement of fluorescence. Addition of 25 to 45 PFU/cell did not increase the effect of 20 PFU/cell. A stepwise addition of phages from 5 to 20 PFU/cell (Fig. 4B) did not increase the relative fluorescence intensity significantly after the first enhancement by 5 PFU/cell (Fig. 4B). The relative intensity of fluorescence was not significantly enhanced when AS-1 was added to cultures containing cells of strains 6307, 6312, and other strains that did not adsorb AS-1. Attachment of AS-1 to cells of strains 6907 or 6911, which adsorb AS-1 at a low rate (Table 2), brought about a small increase of fluorescence (MOI, 12 PFU/cell). The same increase was observed when ghosts of AS-1 were added to cells of *A. nidulans* KM.

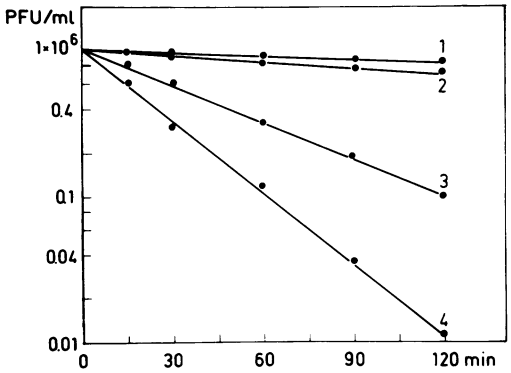



FIG. 3. *Influence of protease treatment on the kinetics of adsorption of cyanophage AS-1 by A. nidulans KM. Treatment of host cells: 3 × 10⁹ cells/ml were treated with 250 μg of protease per ml in Tris-hydrochloride buffer, 0.01 M (pH 7.3), for 15 min at 30° C. The treated cells were washed twice with TMN buffer, resuspended in TMN buffer, and infected by AS-1 at an MOI of 0.01. 1, Adsorption after pretreatment of cells with Pronase; 2, with trypsin; 3 and 4, controls without pretreatment: 3—0.01 M NaCl, 4—0.1 M NaCl.*

**Role of LPS in the attachment process of AS-1.** The exposed position of LPS on the cell surface of unicellular cyanobacteria (11, 20, 40) suggests a role for them as cyanophage receptor. To test this hypothesis, preparations of LPS material isolated from cultures of cyanobacteria were examined for capacity to inactivate cyanophage AS-1. The results show that LPS of typical host strains (Table 2) inactivates the viruses at low concentrations (Table 4). The effect increased linearly as a function of the dosage (Fig. 5). Washing the phage suspension with TMN buffer stopped further inactivation but did not reactivate the phage. Similar dosage response curves were obtained with LPS of the other host strains (6311, 6908, IUCC 1549, IUCC 1191, and *A. nidulans* KM). LPS isolated from strains that do not adsorb AS-1 inactivated AS-1 very slowly (Fig. 5). A five- to sixfold-higher dosage was necessary to obtain an effect comparable to that of host strain LPS (Table 4).

TABLE 3. *Inactivation of AS-1 virus by proteases*

| Enzyme | Decrease (%) of 1.45 × 10⁵ PFU/ml after 60-min treatment at 30°C with protease at: | |
|---|---|---|
| | 2 μg/ml | 10 μg/ml |
| Pronase | 9 | 49 |
| Subtilisin | 4 | 17 |
| Trypsin | 5 | 28 |
| Trypsin + phenylmethylsulfonylfluoride | 0.7 | 2.6 |
| Chymotrypsin | 2.6 | 26 |

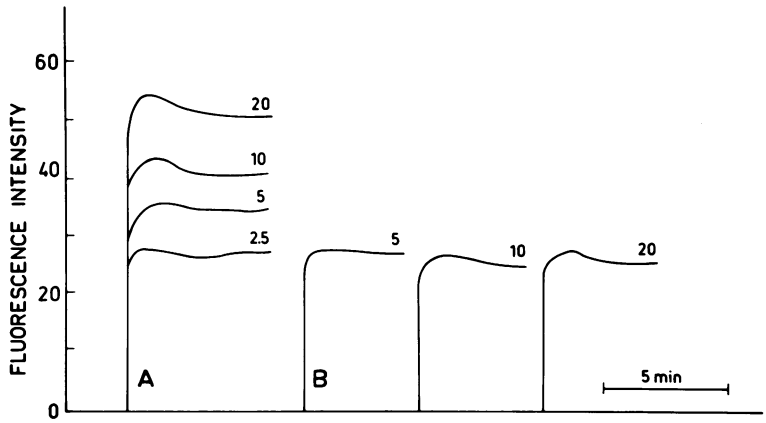



FIG. 4. *Enhancement of fluorescence of ANS-labeled cells by addition of cyanophage AS-1. The base line is the fluorescence of the suspension of Synechococcus 6311. Experimental details are described in the text. (A) Four separate experiments with constant cell density but various concentrations of AS-1. The mean number of PFU per cell is shown on the curve. (B) One experiment. The mean number of PFU per cell was increased stepwise from 5 to 10 to 20 PFU/cell.*

TABLE 4. *Influence of LPS on cyanophage AS-1*

| Material | Serological cross-reaction with antiserum against *A. nidulans* KM | $I_{50}$[a] (μg/ml) |
|---|---|---|
| LPS from: | | |
| *A. nidulans* KM | +++ | 5.4 |
| 6910 | +++ | 5.8 |
| 6908 | +++ | 6.2 |
| 6311 | +++ | 5.4 |
| 6907 | + | 25.0 |
| 6911 | +/− | 20.0 |
| 6307 | − | 34.0 |
| Hydrolyzed | | |
| LPS of *A. nidulans* KM[b] | + | 10.0 |
| LPS fraction II[c] | − | 15.0 |
| Lipid A from LPS of *A. nidulans* | − | 150.0 |

[a] $I_{50}$, Dose required to inactivate 50% of 100 PFU/ml in 60 min.

[b] 1% acetic acid, 5 h, 100°C.

[c] Mannose-enriched carbohydrate fraction from LPS fraction of *A. nidulans* (20).

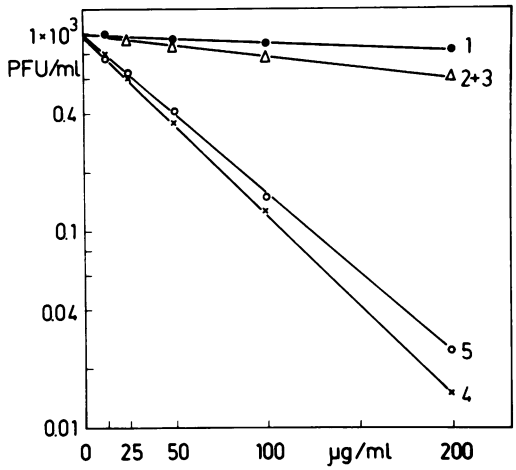



FIG. 5. *Dosage response curve for inactivation of cyanophage AS-1 by LPS of different cyanobacteria. AS-1: 1,000 PFU/ml. Ordinate: PFU per milliliter after incubation of phages with LPS. Abscissa: Dosage of LPS used in the test. Incubation: 120 min, 30°C. LPS from strains 6307 (1), 6907 (2), 6911 (3), 6910 (4), and 6311 (5).*

LPS from strains 6907 or 6911, which adsorbed AS-1 at a low rate, inactivated AS-1 with correspondingly low efficiency. Approximately 70 μg of LPS per ml from strains 6907 or 6911 was needed to inactivate 100 PFU of AS-1 in 2 h.

The amino acid content of the purified LPS of *A. nidulans* amounted to 0.5% of the dry weight. This low concentration seems to exclude the possibility that the traces of protein present in the LPS are responsible for receptor activity. Accordingly, trypsin and Pronase treatment of LPS did not influence the inactivating capacity.

Antibodies against heat-treated (100°C, 2 h) whole cells of *A. nidulans* KM reacted strongly with LPS of cyanobacteria of group Ia (DNA of 50 mol% G+C; Table 4). LPS preparations that show serological cross-reactivity inactivated the cyanophage AS-1 efficiently (Fig. 5; Table 4). The inactivation of virus by LPS was temperature dependent (Fig. 6).

It has been known for some time that LPS in water forms micelles. When the LPS micelles were homogenized by ultrasonication or when the solubility of LPS was increased (up to 10 mg/ml) by electrodialysis and subsequent formation of the triethylamine sodium salt, the capacity to inactivate AS-1 was increased by approximately 15 to 20%.

It has been shown that bacteriophages inactivated by LPS from *E. coli* react specifically with either the O-specific side chains of LPS or the core region (24). Since no mutants of *A. nidulans* with a rough-type LPS were available, we tested fractions of degraded LPS for their efficiency to inactivate AS-1. The LPS was partially degraded by hydrolysis (10% acetic acid, 3 h, 100°C [20]). The lipid A fraction of the hydrolysate was separated from the polysaccharide moiety by centrifugation, and the carbohydrate fractions were separated by chromatography on Sephadex G50 (20). Lipid A did not inactivate AS-1 (Table 4). The hydrolysate of LPS from *A. nidulans* KM and the mannose-enriched carbohydrate fraction from this hydrolysate have a lower inactivating capacity in comparison with untreated LPS (Table 4). The chemical studies on the structure of *A. nidulans* KM LPS (20) and the studies with different degraded fractions do not, however, allow conclusive identification of a specific region of the LPS moiety involved in the reaction with the tail of AS-1.

**Isolation of receptor material.** Although the preceding experiments suggest the participation of LPS in the attachment process, the protease effect seems to indicate that proteins also participate in the adsorption process. For this reason, protein material was extracted from the cell surface and examined for receptor activity. The extraction with 0.1 N NaOH (10) gave the best results. The extraction with detergents such as sodium cholate (27), sodium dodecyl sulfate, or Triton X-100 solubilized substantial amounts of pigment and other intracellular material. Extraction with EDTA in buffer had little effect (Table 5). Ten to 15 μg (dry weight) of the alkaline extract inactivated 50% of 100

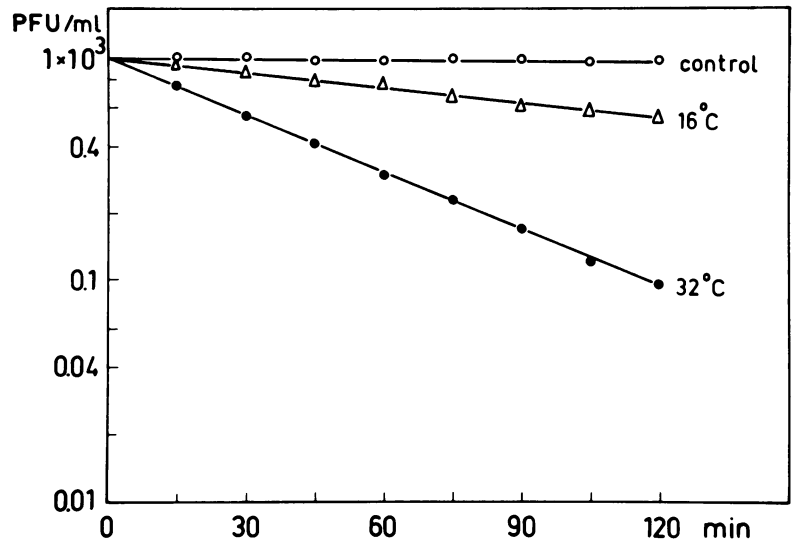



FIG. 6. *Effect of temperature on the kinetics of inhibition of phage AS-1 by LPS of S. cedrorum IUCC 1191. Symbols: (○) control—AS-1 in TMN buffer without LPS at 30°C; (△) AS-1 (1,000 PFU/ml) incubated at 16°C with 150 μg of LPS per ml; (●) incubation at 32°C. After various periods of incubation of AS-1 with LPS, the number of PFU per milliliter was determined.*

TABLE 5. *Yield and activity of material extracted from A. nidulans*

| Method of extraction | Yield of receptor activity (%, dry wt) extracted from whole cells at: | | $I_{50}$[a] (μg/ml) |
|---|---|---|---|
| | 20°C | 40°C | |
| 0.1 N NaOH (ref. 10) | 1.8 | 2.0 | 6.5 |
| 0.1 N NaOH, 1% SDS[b] | 2.5 | 2.9 | 8.1 |
| SDS, 1% | 0.9 | 1.5 | 6.2 |
| SDS, 2% | 2.1 | 3.7 | 6.6 |
| 5 mM EDTA | 0.07 | 0.2 | 71.4 |
| Sodium cholate (2%) in 5 mM EDTA | 2.1 | 3.2 | 7.5 |
| Triton X-100 | 2.3 | 3.3 | 7.5 |

[a] $I_{50}$, Dose required to inactivate 50% of 100 PFU/ml in 60 min.

[b] SDS, Sodium dodecyl sulfate.

PFU/ml in 60 min ($I_{50}$). The proteins of this extract were precipitated by ammonium sulfate and, after dialysis, were applied to Bio-Gel A50 columns (Fig. 7). Fraction III of this chromatography still contained LPS but was free of biliproteins and other inactive proteins. Subsequent chromatography on DEAE-cellulose reduced the carbohydrate content of the receptor-active fraction A to 6% (Fig. 8). This fraction A was then subjected to chloroform-methanol extraction (27). The highest activity was found in the methanol phase ($I_{50}$ = 2.7 μg/ml). In this fraction, three major polypeptides with apparent molecular weights of 54,000 to 64,000 and two minor bands with a molecular weight of approximately 30,000 were detected by polyacrylamide gel electrophoresis. A complete purification of proteins from LPS was achieved neither by these methods nor by density gradient centrifugation and electrophoresis on cellogel.

Although the receptor material in the alkaline extract was freed of phycobiliproteins and glycolipids and enriched in three major proteins, the receptor activity based on dry weight did not increase in the same ratio (enrichment approximately sevenfold). This could be interpreted as an indication that LPS is the only material with a receptor function. A degradation of the proteins in the active fraction by trypsin, however, caused a decrease in receptor activity (Table 6). Furthermore, purified protein material with receptor activity was found to be more active than LPS (Fig. 9 and 10).

Electron microscopic studies have shown that the tail sheath of phage AS-1 particles treated with receptor material from *A. nidulans* KM is contracted and the phage head is empty (Fig. 11 and 13). A comparable tail contraction was not observed when AS-1 was treated with cell wall extracts of *Coccochloris peniocystis* 6307 (Fig. 12).

## DISCUSSION

It has been previously reported that *Synechococcus* strains 6301, 6311, and 6908 of typological group Ia (35) are susceptible to cyanophage AS-1 (29; Safferman and Morris, Abstr.). We have found that the host range of AS-1 is restricted to those strains of typological group Ia that have DNA with approximately 50 mol% G+C (Table 2). The ability of AS-1 to infect cells of these

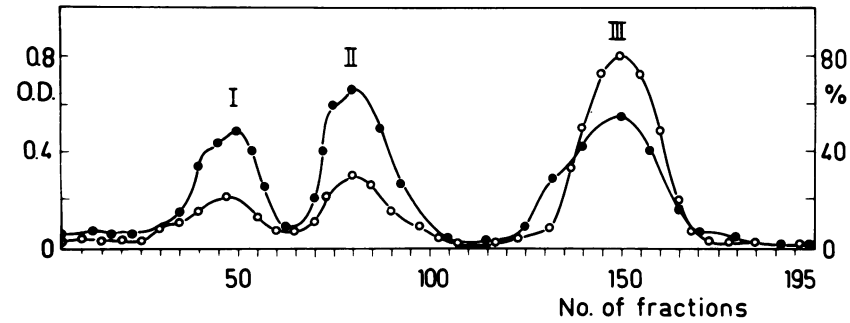



FIG. 7. *Chromatography of alkaline extract from A. nidulans KM on Bio-Gel A50. Column (90 by 3 cm) was eluted with 10 mM sodium phosphate buffer, pH 7.0, at 20 ml/h. Fractions I and II contained pigment (biliproteins) and LPS. Fraction III contained 40% (dry weight) of the alkaline extract and 83% of the activity. It consisted of 76% protein and 24% carbohydrate. Symbols: (○) receptor activity; (●) protein concentration.*

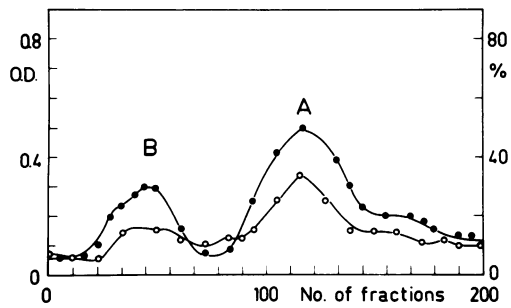



FIG. 8. *Chromatography of fraction III from Bio-Gel on DEAE-cellulose (see text). Fraction A contained 74% of the total receptor activity applied to the column and fraction B contained 17%. Fraction A contained 6.6% sugars (5.6% mannose); fraction B contained 66.5% total sugars (46% mannose). Symbols: (○) receptor activity; (●) protein concentration.*

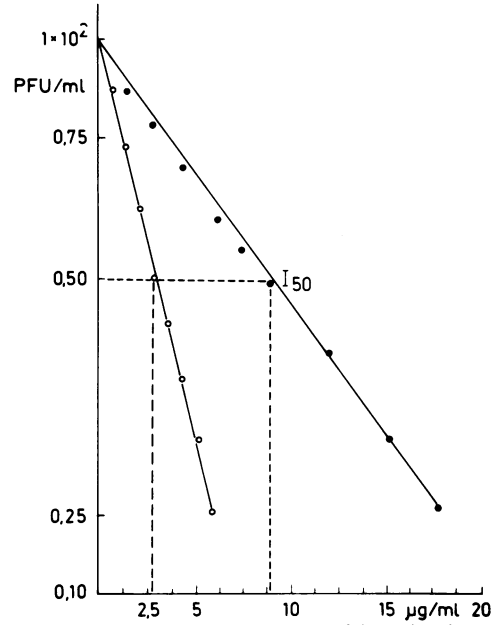



FIG. 9. *Dosage response curve of inactivation of cyanophage AS-1 by an extract of cell surface proteins enriched in receptor activity (○) or LPS (●) from A. nidulans KM. $I_{50}$: Dosage of material that inactivated 50% of 200 PFU/ml at 30°C in 60 min.*

strains is correlated with a characteristic LPS composition (12, 20; Schmidt, personal communication).

The LPS of typical host strains contain a mannose polymer in addition to fucose, glucose, galactose, 3-*O*-methyl-D-mannose, 4-*O*-methyl-D-mannose, and 2-keto-3-deoxyoctonate as characteristic sugar components (12, 20). In contrast, strains with DNA of a high G+C ratio do not adsorb AS-1 and have a significantly different LPS composition (12). The LPS of the non-host strain 6307 (Table 2) contains rhamnose as a major sugar component and fucose, glucose, galactose, mannose, and tyvelose (3,6-dideoxy-D-mannose) but no 2-keto-3-deoxyoctonate (12; Schmidt, personal communication). Cells of strains 6907 and 6911, however, can adsorb AS-1 with a low rate and are lysed but do not propagate AS-1 (Table 2). The sugar composition of the LPS of strain 6911 is the same as that of strain 6307. The sugar pattern in the LPS of strain 6907, however, is comparable to that of typical host strains (Schmidt, personal communication). The low serological cross-reactivity of LPS from strains 6307, 6907, and 6911 with antiserum against cells of *A. nidulans* KM (Table 4) indicates structural differences in these LPS in comparison with LPS of typical host strains. We have been unable to detect mutants of host strains resistant to infection by phage AS-1 that have defects in the carbohydrate region of LPS.

Isolated LPS as well as isolated protein material of typical host strains inactivate AS-1 (Fig. 5, 9, 10). The purified protein material from *A. nidulans* KM is not entirely free of LPS. It

TABLE 6. *Effect of trypsin on activity of the receptor complex*[a]

| Sample | Protein content of fractions (μg/ml) | | Dose of receptor (μg/ml) for 50% inactivation of AS-1 in 2 h | |
|---|---|---|---|---|
| | Before trypsin treatment | After trypsin treatment | Before trypsin treatment | After trypsin treatment |
| Alkaline extract | 68 | 5 | 9.6 | 32.2 |
| Active fraction from DEAE chromatography | 80 | 14 | 5.3 | 26.5 |
| Active fraction from DEAE chromatography, chloroform-methanol extraction, and $(NH_4)_2SO_4$ precipitation | 78 | 2 | 2.0 | 27.7 |
| Material purified as before, electrophoresis on cellogel | 88 | 8 | 10.7 | 30.5 |

[a] See Materials and Methods.

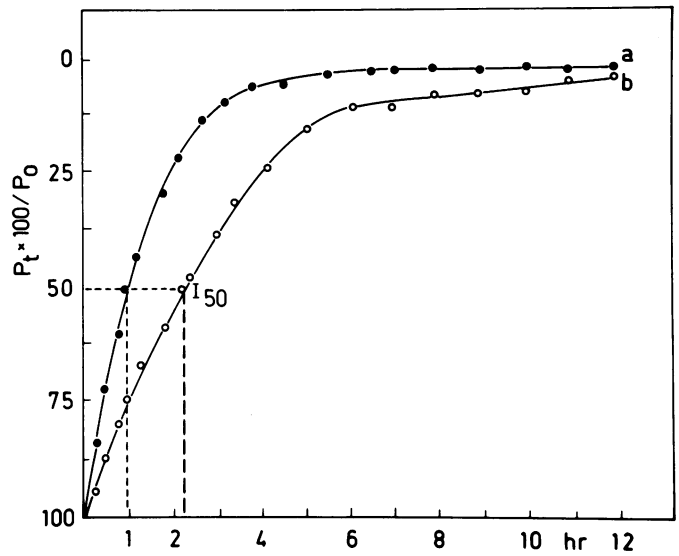
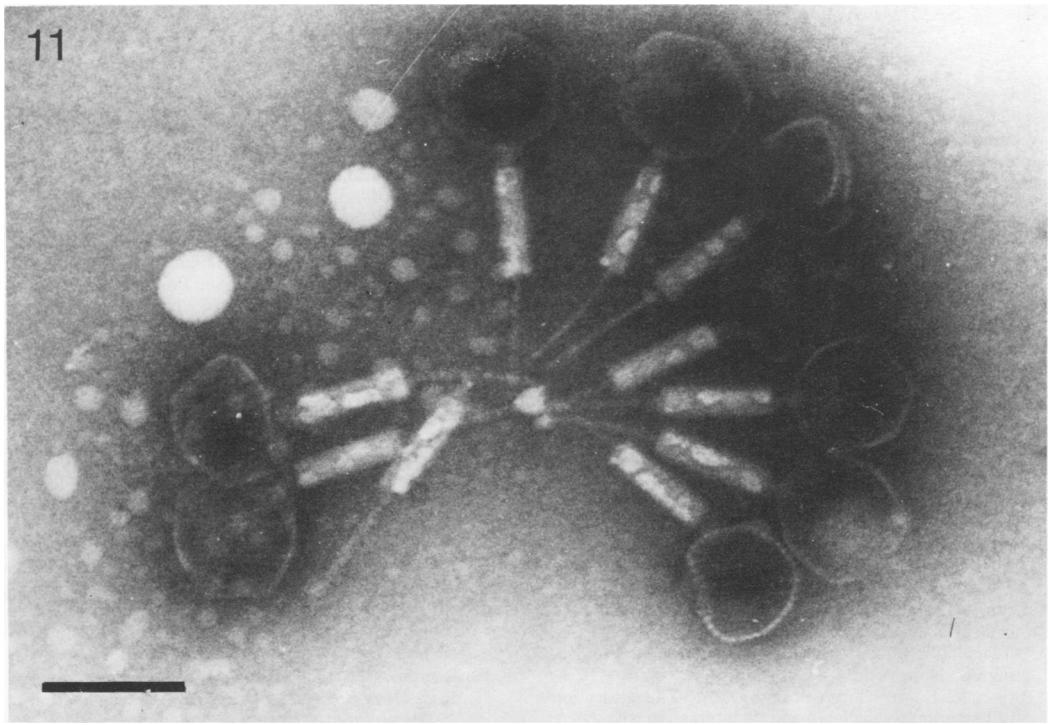
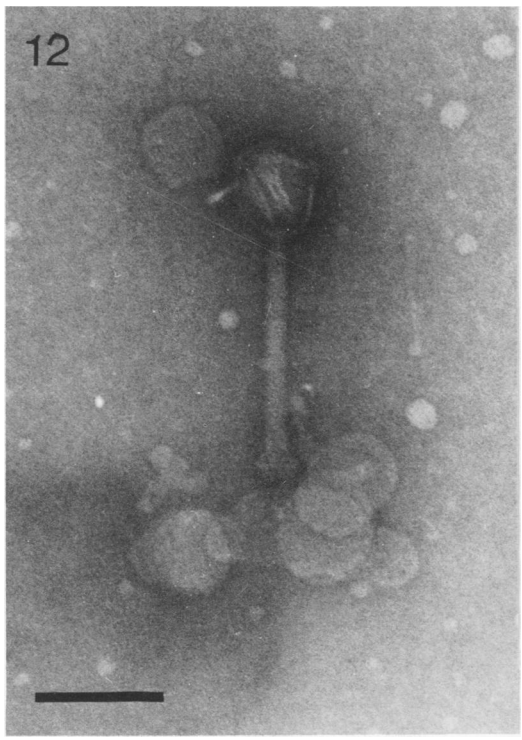



FIG. 10. *Kinetics of inactivation of cyanophage AS-1 by receptor material (a) and LPS (b) from A. nidulans KM. $P_t$, PFU per milliliter at time t (abscissa); $P_0$, PFU per milliliter at time 0. Ordinate: Percentage of active phages (PFU) after incubation of AS-1 with inhibitor material. PFU per milliliter at $t_0$ = 1,500. Concentration of LPS and receptor material (purified protein fraction from alkaline extract) = 150 μg/ml.*

contains about 5% carbohydrates. The major proteins of this fraction have molecular weights in the range 54,000 to 64,000. Isolated cell walls of *A. nidulans* KM contained 31 polypeptides, including five major proteins, of which two banded in the same region of the polyacrylamide gel as the receptor-active material (15). A comparison of dosage response curves for LPS and protein material (Fig. 10) excludes the possibility that small amounts of LPS in the protein material (≤5%) or traces of protein in the LPS fractions (≤0.5%) are responsible for the respective inactivating capacities. Extracts of non-host strains show only a very low activity. The effect of proteases, both on the surface of intact cells and on isolated receptor material, provides further evidence for a participation of proteins in the infection process.

We observed that during the purification of proteins from the alkaline extract, the specific activity ($I_{50}$) does not increase in the same ratio in which specific proteins are enriched. The reason for relative loss of activity is as yet unknown, but might be due to the degradation of molecular organization during isolation. It is possible that the LPS of host strains are responsible for the first steps of recognition, enabling then the attached phage to move laterally over the surface of the outer membrane (25) to a final position over an adhesion zone of the cytoplasmic and outer membranes (6), where a series of irreversible events take place culminating in the injection of DNA into the cells. The receptor complex may contain proteins that span the outer membrane. Alternatively, both LPS and protein are involved in the process of attachment.



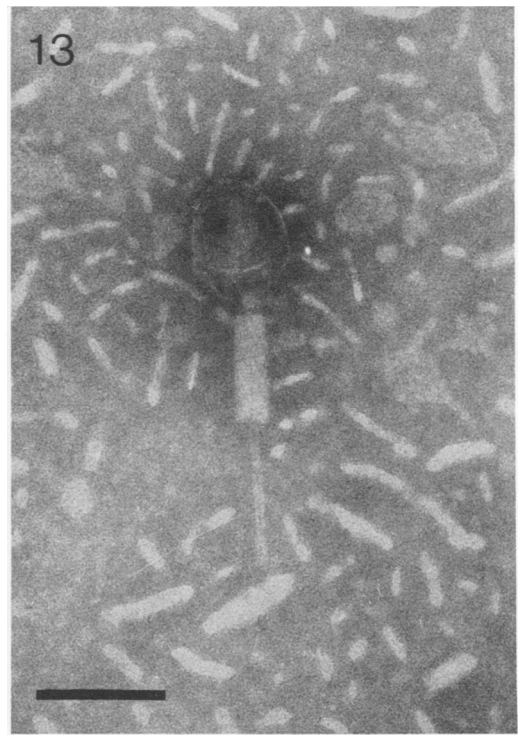




FIG. 11. *Inactivation of cyanophage AS-1 by purified protein material from cell surface of A. nidulans. The heads of phages are empty and the tail sheaths are contracted. Negative staining by phosphor-tungstic acid (2%). Bar = 0.1 μm.*

FIG. 12. *Reversible attachment of cyanophage AS-1 to purified protein material from cell surface of the non-host strain C. peniocystis 6307. Bar = 0.1 μm.*

FIG. 13. *Inactivation of cyanophage AS-1 by LPS-enriched material (fraction I from chromatography of alkaline extract from A. nidulans KM; Fig. 7). Bar = 0.1 μm.*

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